17. Individually caged mice had free access to standard Chow and were treated in accordance with Stanford guidelines. At 11 weeks of age, female F_0 animals weighed 30.5, 35.5, and 41.9 g, and male F_0 animals weighed 32.4, 34.8, and 43 g—significantly more than nontransgenic littermates (females: 21.1 ± 2.0 g, n = 10, P = 0.02; males: 26 ± 2.0 g, n = 5, P = 0.03, student's t test). At 15 weeks of age, body length of F_1 transgenic animals (10.5 ± 0.1 cm, n = 4) was more than that of nontransgenic littermates (9.18 ± 0.2 cm, n = 10, P = 0.0002). Food consumption measured over a 7-day period at 12 weeks of age for F_1 transgenic animals (27.9 ± 5.4 g, n = 4) was more than that of nontransgenic littermates (21.9 ± 2.8 g, n = 5, P = 0.03).
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NF-AT Activation Induced by a CAML-Interacting Member of the Tumor Necrosis Factor Receptor Superfamily

Götz-Ulrich von Bülow and Richard J. Bram*

Activation of the nuclear factor of activated T cells transcription factor (NF-AT) is a key event underlying lymphocyte action. The CAML (calcium-modulator and cyclophilin ligand) protein is a coinducer of NF-AT activation when overexpressed in Jurkat T cells. A member of the tumor necrosis factor receptor superfamily was isolated by virtue of its affinity for CAML. Cross-linking of this lymphocyte-specific protein, designated TACI (transmembrane activator and CAML-interactor), on the surface of transfected Jurkat cells with TACI-specific antibodies led to activation of the transcription factors NF-AT, AP-1, and NFκB. TACI-induced activation of NF-AT was specifically blocked by a dominant-negative CAML mutant, thus implicating CAML as a signaling intermediate.

We identified proteins that can interact with CAML in a two-hybrid screen (*1*, *2*). To determine if any of these CAML-binding proteins affected signaling in T cells, we examined their ability to modulate activity of the Ca^{2+}-dependent transcription factor NF-AT (*3*). Overexpression of the two-hybrid clones in Jurkat T cells revealed that expression of one clone (encoding the TACI protein) led to activation of NF-AT, suggesting that TACI may lie in the same signaling pathway as CAML. The deduced amino acid sequence of TACI (Fig. 1A) (*4*) includes a single hydrophobic region (residues 166 to 186) that has features of a membrane-spanning segment. Analysis of the protein sequence (*5*) predicted extracellular exposure for the NH_2-terminus with a cytoplasmic COOH-terminus. Although TACI lacks an NH_2-terminal signal sequence, the presence of an upstream stop codon indicates that the complete open reading frame is contained within the clone (*6*). The predicted cell-surface location of TACI was confirmed in intact Cos-7 cells transfected with an expression plasmid encoding TACI with an NH_2-terminal FLAG epitope tag. Staining with monoclonal antibody to FLAG revealed TACI localized to the cell surface (Fig. 2A). TACI is therefore a type III transmembrane protein with an extracellular NH_2-terminus in the absence of a cleaved signal sequence (*7*). Inspection of the TACI protein sequence also revealed two repeated regions (residues 33 to 66 and 70 to 104) that are 50% identical. A PROSITE motif search (*8*) identified this repeated region as a cysteine-rich motif characteristic of the tumor necrosis factor receptor (TNFR) superfamily. Comparison of TACI with other members of TNFR superfamily (Fig. 1B) demonstrates the similarity between these domains, with the best match to cysteine-rich domains of DR3 (also known as Wsl-1, Apo-3, or TRAMP) (*9*).

Northern blot analysis of TACI mRNA demonstrated a 1.4-kb transcript expressed in spleen, small intestine, thymus, and peripheral blood lymphocytes, suggesting that a single TACI transcript is present in both T and B lymphocytes (Fig. 2B). Specific antibody staining of peripheral blood cells with a polyclonal antibody to TACI (*10*) revealed the presence of TACI on the surface of B cells, but not resting T cells (Fig. 2C). Because expression of other TNFR members such as CD30 is increased after activation of T lymphocytes (*11*) and because TACI appears to be expressed in thymocytes, we examined T cells activated with ionomycin and phorbol ester. Such treatment of T cells induced the synthesis of cell-surface TACI in 54% of CD2-positive cells within 48 hours (Fig. 2D). This subset was equally distributed between CD4 and CD8 cells. Stimulation of interleukin-2 (IL-2)–dependent T cells with antibodies to CD3 and CD28 also induced expression of TACI. A reverse transcriptase–polymerase chain reaction assay revealed TACI message in resting B cells but not in T cells, unless they were activated (*6*).

Neither TACI mRNA nor protein could be detected in untransfected Jurkat cells expressing the SV40 large T-antigen (TAg), either unstimulated or treated with phorbol myristyl acetate (PMA) and ionomycin (*6*). To assess the effect of TACI on NF-AT activity in T cells, we transiently expressed the protein in TAg Jurkat cells along with a secreted alkaline phosphatase reporter driven by the NF-AT–binding sequences from the IL-2 promoter (*12*, *13*, *14*). TACI overexpression could partially replace the requirement for PMA and ionomycin in this assay for maximal activation of the NF-AT reporter. The addition of antibodies to TACI to the cells increased NF-AT activation up to sevenfold (Fig. 3A), demonstrating that TACI responds to cross-linking at the cell surface. This affinity-purified antibody to TACI had no effect on control transfected cells. To further verify the specificity of the response, we transfected cells with an NH_2-terminal FLAG-epitope–tagged TACI expression plasmid and incubated them with the M2-FLAG monoclonal antibody (*15*). This treatment gave a similar increase in NF-AT activity (Fig. 3A). The degree of NF-AT activation varied among different experiments because of transfection efficiency, but was typically 40 to 100% of the maximal re-

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sponse to PMA plus ionomycin.

Activation of NF-AT in T cells requires the activation of both the calcium-dependent protein phosphatase calcineurin and the AP-1 transcription factor (*16*). Overexpression of CAML in TAg Jurkat cells activates calcineurin, and stimulation with PMA is required to activate NF-AT (*12*). NF-AT activation by TACI did not depend on additional stimulation with PMA, suggesting that AP-1 may also be activated upon TACI ligation. Reporter assays with the mouse metallothionine AP-1–binding sequence confirmed this (Fig. 3B). Furthermore, ligation of TACI also activated NFκB (Fig. 3C), a transcription factor implicated in the actions of other members of the TNFR superfamily (*17*). TACI-mediated activation of NF-AT depended on calcineurin, as demonstrated by the loss of NF-AT activity in the presence of immunosuppressive drugs such as cyclosporin A (CsA) (Fig. 3D) or FK506. Depletion of external calcium also blocked TACI-induced NF-AT activation (Fig. 3E).

We determined the regions of TACI and CAML required for their interaction, using deletion mutants with the yeast two-hybrid system (*2*). The COOH-terminal 126 amino acids of TACI were sufficient to bind to the NH2-terminal 201 amino acids of CAML (Fig. 4A). Evidence for the association of TACI with CAML in vivo was provided by experiments in which full-length CAML or the 201 NH_2-terminal amino acid residues of CAML coimmunoprecipitated with TACI from lysates of cells overexpressing both proteins (Fig. 4B). We conclude that the cytoplasmic COOH-terminal portion of TACI can physically associate with the NH_2-terminal half of CAML.

CAML is a widely expressed integral membrane protein localized to cytoplasmic vesicles (*12*). Hydrophobic domains in the

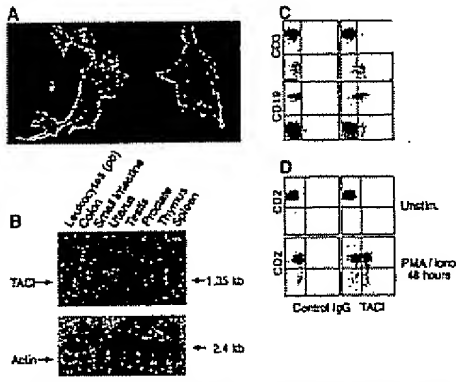

Fig. 2. Cell-surface expression of TACI in some B and T lymphocytes. (A) Cos-7 cells transiently transfected with a plasmid encoding NH_2-terminal FLAG-tagged TACI. The NH_2-terminal epitope tag appeared to be extracellular, because specific staining with the antibody was observed even in the absence of permeabilizing detergent. (B) Expression of TACI in lymphoid cells. Multiple-tissue Northern (RNA) blot was probed with TACI cDNA. TACI mRNA was not detected in heart, brain, placenta, lung, liver, skeletal muscle, kidney, or pancreas. pb indicates peripheral blood. (C) Shown is flow cytometric analysis of fresh peripheral blood lymphocytes stained with antibodies specific for either B cells (CD19) or T cells (CD3, *y* axes) and rabbit IgG control antibodies or antibodies to TACI (*x* axes), as indicated. (D) Induction of TACI expression in a subset of activated T cells. Flow cytometry is shown for peripheral blood T lymphocytes (*23*) cultured in the absence or presence of PMA (25 ng/ml) and ionomycin (1 μM) for 48 hours, and stained with anti-CD2 (T cell–specific, *y* axes) and control IgG or anti-TACI (*x* axes), as indicated. PMA and ionomycin treatment induced surface TACI expression in 54% of CD2-positive T cells. Unstim., unstimulated; Iono, ionomycin.

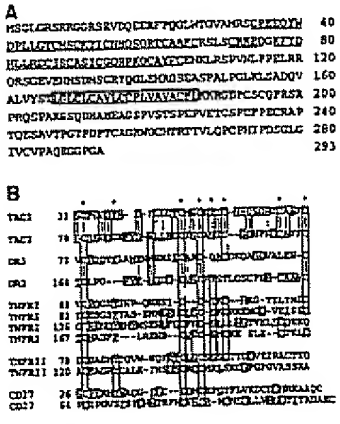

Fig. 1. Structural features of TACI. (A) Predicted amino acid sequence. Residues composing the proposed transmembrane domain are boxed, and the cysteine-rich TNFR repeats are underlined. (B) Alignment of the cysteine-rich repeats from TACI with those from some members of the TNFR superfamily (*9*, *21*). Boxed letters indicate residues from other receptors that share identity with the TACI protein; underlined residues are conservative substitutions. Asterisks denote conserved cysteines and other residues that stabilize the conformation of TNFRI (*22*).

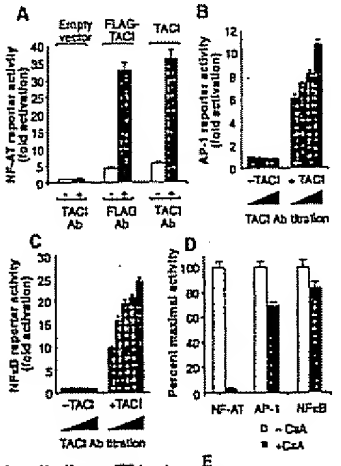

Fig. 3. Activation of NF-AT, AP-1, and NFκB-specific transcription in cells with stimulated TACI. (A) Activation of an NF-AT–driven secreted alkaline phosphatase (SEAP) reporter. NF-AT-specific activity was determined in TAg Jurkat cells, cotransfected with the SXNFAT reporter (*14*) and the expression plasmid pBJ5 (*24*) containing no insert, cDNA encoding NH_2-terminal FLAG-epitope-tagged TACI, or native TACI, in the absence or presence of cross-linked monoclonal antibody to FLAG or polyclonal antibodies to TACI, as indicated. Data were corrected for transfection efficiency, using the maximal response to PMA and ionomycin. Fold-activation ratios were then calculated as compared with the empty vector–transfected, unstimulated control value. (B) Activation of AP-1–specific transcription. TAg Jurkat cells were cotransfected with a mouse metallothionine AP-1–SEAP reporter plasmid (*14*) and pBJ5 containing no insert (−TACI, left) or TACI cDNA (+TACI, right). Cells were incubated with various amounts of antibodies to TACI. To control for transfection efficiency, we included a plasmid containing a constitutive promoter driving the expression of luciferase (EF-Luc). (C) Activation of an NFκB-specific transcription. The experiment was performed as in (B) except that an NFκB-specific SEAP reporter was used. (D) Inhibition of TACI-mediated activation of NF-AT by CsA. TAg Jurkat cells were transfected with a TACI expression plasmid and reporter plasmids specific for NF-AT, NFκB, or AP-1. Activation after treatment with antibodies to TACI was determined in absence (−) and presence (+) of CsA (100 ng/ml). (E) Requirement of extracellular Ca^{2+} for NF-AT activation in response to antibody cross-linked TACI. NF-AT activation in TAg Jurkat cells expressing TACI was measured in the presence of EGTA to remove extracellular calcium. The treatments included cross-linked antibody to TACI, transfection with a COOH-terminally truncated, calcium-independent calcineurin A subunit, or activation with the OKT3 antibody to the T cell receptor. All cells were also stimulated with PMA (50 ng/ml).

COOH-terminal half of the protein are essential for activity, and the hydrophilic NH_2-terminal half of the protein may have a regulatory role (18). The NH_2-terminal half of the molecule is exposed on the cytoplasmic surface of the vesicle (19). To test whether TACI might signal by directly interacting with CAML, we investigated the possibility that the interacting domain of CAML (residues 1 to 201) could inhibit TACI-induced activation of a transcription factor. Cotransfection of the mutant CAML(1-201) expression plasmid CLX91 inhibited TACI-induced activation of NF-AT by more than 80% (Fig. 4C). There was no inhibitory effect on NF-AT activity induced by PMA and ionomycin, thus ruling out a nonspecific toxic effect of the mutant CAML protein. Coexpression of CAML(1-201) also did not reduce the accumulation of TACI protein, as detected by protein immunoblotting (Fig. 4C), nor did flow cytometric comparison of cells transfected with TACI and CAML(1-201) expression plasmids or with the TACI plasmid and empty vector reveal any differences in surface accumulation of TACI (6). The CAML mutant had little effect on the activation of AP-1 or NFκB in response to ligation of TACI (Fig. 4D).

Overexpression of CAML in T cells contributes to calcium-dependent constitutive activation of NF-AT. We identified a lymphocyte-specific receptor with characteristics of the TNFR superfamily that can activate NFκB, NF-AT, and AP-1. The signal from TACI to NF-AT activation may proceed through direct interaction between TACI, at the cell surface, and CAML, an integral membrane protein located at intracellular vesicles.

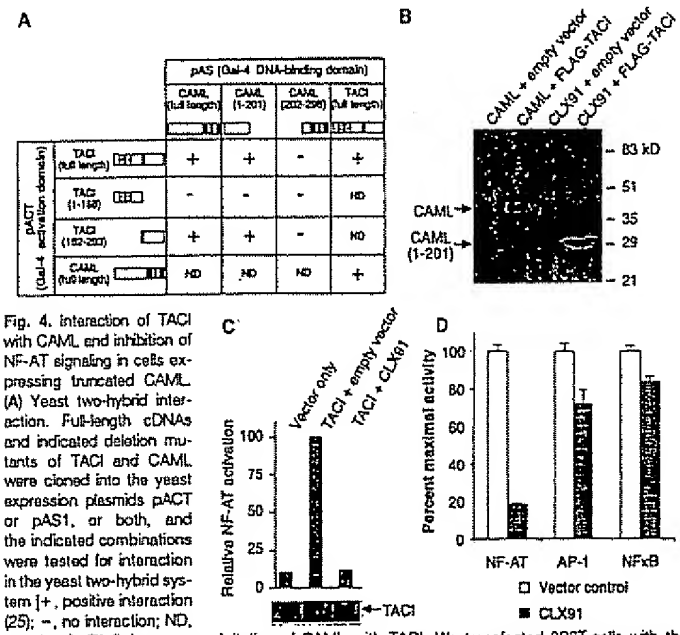

Fig. 4. Interaction of TACI with CAML and inhibition of NF-AT signaling in cells expressing truncated CAML. (A) Yeast two-hybrid interaction. Full-length cDNAs and indicated deletion mutants of TACI and CAML were cloned into the yeast expression plasmids pACT or pAS1, or both, and the indicated combinations were tested for interaction in the yeast two-hybrid system [+, positive interaction (25); -, no interaction; ND, not done]. (B) Coimmunoprecipitation of CAML with TACI. We transfected 293T-cells with the indicated combinations of the expression plasmid pBJ5 containing cDNAs for CAML, TACI with an NH_2-terminal FLAG tag, the NH_2-terminal 201 amino acids of CAML (CLX91), or no insert. After 48 hours of incubation, the cells were lysed [1% dodecyl maltoside, 20 mM Hepes (pH 7.4), 150 mM NaCl, 10% glycerol, 2 mM $MgSO_4$, 1 mM $CaCl_2$, and 1 mM phenylmethylsulfonyl fluoride], and the lysate was clarified by centrifugation. FLAG-tagged TACI and associated proteins were immunoprecipitated with monoclonal antibody to FLAG conjugated to agarose beads and subjected to protein immunoblotting. The blot was probed with immunoaffinity-purified polyclonal antibodies to CAML (10) followed by chemiluminescent detection (Amersham). Parallel protein immunoblots of each sample confirmed the expected expression of TACI, CAML, or the truncated CAML mutant in all transfections. (C) Inhibition of TACI-induced NF-AT activation in cells overexpressing the NH_2-terminal half of CAML. NF-AT activation in cells treated with an antibody to TACI was determined in TAg Jurkat cells transfected with pBJ5 alone, pBJ5-TACI plus a vector control, or pBJ5-TACI with an equivalent amount of CLX91 (top). CLX91 did not alter levels of TACI expression in transfections, as determined by protein immunoblotting with polyclonal antibodies to TACI (bottom). (D) Specificity of the dominant-negative effect of the truncated CAML mutant. Antibody-induced TACI activation of NF-AT, AP-1, and NFκB reporter activities were determined by transient transfection in TAg Jurkat cells. Cells were cotransfected with CLX91 or vector controls in the presence of pBJ5-TACI and reporter plasmids. Data were normalized against values obtained for stimulation with PMA and ionomycin.

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Evidence for a Role of CRM1 in Signal-Mediated Nuclear Protein Export

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Chromosome maintenance region 1 (CRM1), a protein that shares sequence similarities with the karyopherin β family of proteins involved in nuclear import pathway, was shown to form a complex with the leucine-rich nuclear export signal (NES). This interaction was inhibited by leptomycin B, a drug that prevents the function of the CRM1 protein in yeast. To analyze the role of the CRM1-NES interaction in nuclear export, a transport assay based on semipermeabilized cells was developed. In this system, which reconstituted NES-, cytosol-, and energy-dependent nuclear export, leptomycin B specifically blocked export of NES-containing proteins. Thus, the CRM1 protein could act as a NES receptor involved in nuclear protein export.

Bidirectional transport across the nuclear envelope occurs through nuclear pore complexes. This process requires specific sequences found within transport substrates, soluble transport proteins, and nucleoporins. Thus, the import of nuclear proteins is governed by different nuclear localization sequences (NLS) that are presumably recognized by distinct receptors (karyopherins, importins, and transportins) that mediate the docking of the transport substrate at the cytoplasmic face of the nuclear pore (*1*) and by other soluble factors, including the small guanosine triphosphatase Ran and p10, that are responsible for the translocation step across the nuclear pore complex (*2*). Recent studies have shown that soluble factors involved in the docking step of nuclear import and in Ran-binding ability share amino acid sequences and structural homology domains. The CRM1 protein shares sequence homology in its NH_2-terminal region with the karyopherin β family, as well as with the Ran–guanosine triphosphate (GTP)–binding domain of the Ran-GTP–binding protein family. This protein, which is encoded by an essential gene in yeast, is located at the nuclear pore complex as well as in the nucleoplasm (*3*, *4*). Thus, we examine whether CRM1 could be involved in nuclear export.

Although most nuclear proteins are potential shuttling proteins (*5*), amino acid sequences responsible for highly efficient nuclear export (NES) have recently been identified in an increasing number of proteins, in particular, the human immunodeficiency virus–type 1 (HIV-1) Rev protein, the protein kinase A inhibitor IkappaBα (IκBα), and the heterogeneous nuclear ribonucleoproteins (hnRNPs) A1 and K (*6*). With the exception of the hnRNPs, NES is a leucine-rich sequence in which leucine residues are critical for targeting proteins out of the nucleus. Molecular mechanisms governing NES-dependent nuclear protein export are less well documented than those of the nuclear protein import pathway. However, the existence of NES as well as its ability to saturate NES-dependent export strongly suggest the involvement of specific NES receptors in this process.

To analyze the role of CRM1 in nuclear protein export, we first tested the ability of human CRM1 protein to bind a leucine-rich sequence (NES) (*7*). For this purpose, interaction of human CRM1 with wild-type IκBα or IκBα-L234, a nuclear export mutant of IκBα in which leucine residues of NES have been replaced by alanine (*6*, *8*), was analyzed. In vitro–translated human MYC-tagged CRM1 was mixed with in vitro–translated SV5-tagged wild-type IκBα or IκBα-L234 before being processed for immunoprecipitation with an antibody to the MYC tag (anti-MYC tag) or an anti-SV5 tag. CRM1 protein and wild-type IκBα coprecipitated with both an-

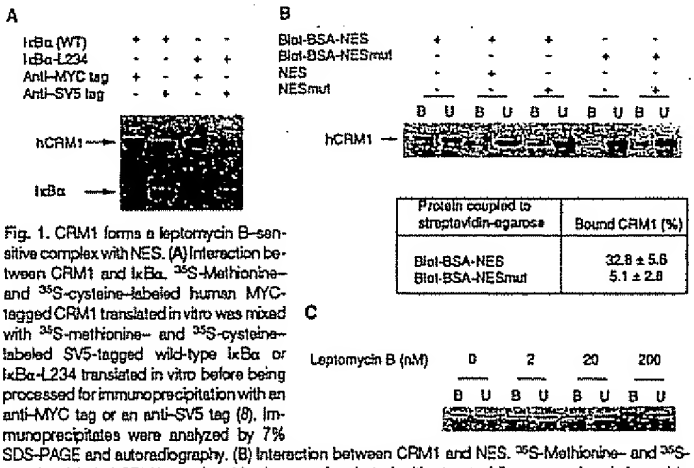

Fig. 1. CRM1 forms a leptomycin B–sensitive complex with NES. (A) Interaction between CRM1 and IκBα. ^{35}S-Methionine– and ^{35}S-cysteine–labeled human MYC-tagged CRM1 translated in vitro was mixed with ^{35}S-methionine– and ^{35}S-cysteine–labeled SV5-tagged wild-type IκBα or IκBα-L234 translated in vitro before being processed for immunoprecipitation with an anti-MYC tag or an anti-SV5 tag (*8*). Immunoprecipitates were analyzed by 7% SDS-PAGE and autoradiography. (B) Interaction between CRM1 and NES. ^{35}S-Methionine– and ^{35}S-cysteine–labeled CRM1 translated in vitro was incubated with streptavidin-agarose beads bound to biotinylated BSA-NES (biot-BSA-NES) or mutated NES (biot-BSA-NESmut) conjugates (*8*). The binding was performed with or without NES or mutated NES (NESmut) peptides (each 2 mg/ml). Bound (*B*) and unbound (*U*) fractions were collected and analyzed by 7% SDS-PAGE and autoradiography. The Bioprint acquisition system and Bioprofil program were used to quantify the autoradiograms. Values were obtained from five independent experiments. (C) ^{35}S-Methionine– and ^{35} S-cysteine–labeled CRM1 translated in vitro was incubated with streptavidin-agarose beads bound to biotinylated BSA-NES conjugate (30 min at room temperature in PBS) with increasing concentrations of leptomycin B. Bound and unbound fractions were collected and analyzed by 7% SDS-PAGE and autoradiography.

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